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## Morbidity scoring after abdominal surgery

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### Mini-abstract

Post-operative monitoring of pain and distress in small rodents is not standardized and widely accepted score sheets are not available. Here we describe a score sheet used in abdominal surgery of rodents, with particular reference to procedures on the liver.

## Introduction

The world-wide use of animals for medical research has resulted in an enormous number of different “disease models” in a few select species, primarily rodents. The focus of these animal models is on the simulation of a particular disease, either through genetic manipulation, application of drugs or surgical manipulations. These manipulations can be a potential source of pain and suffering. The protection of animals used for scientific purposes is regulated by country specific laws, but the way these laws are implemented shows considerable variation between the countries.

Despite the lack of standardized animal protection laws, the development and use of animal models should not only focus on the simulation of disease but also include an evaluation of proper pain management and surveillance. A lot of efforts have been invested in the past to develop reliable protocols for the assessment of pain and analgesic treatments of laboratory animals.

Nevertheless, mainly due to the uncertainty regarding pain and stress detection and incongruences in the data obtained, no final protocols could be developed yet for appropriate and practicable monitoring and management of pain and suffering.

Current efforts into improved behavioral monitoring and pain assessment clearly indicate a need for action. As an example, the use of the opioid buprenorphine for post-operative analgesia is widely accepted.<sup>1</sup> Although its half-life is longer than in other opioid-analgesics, it is less than four hours, requiring a tight and repetitive application every 4-6 hours by injection. It is obvious that the investigator doesn't want to follow such a schedule as it would indicate an application of analgesia twice during the night. Also, repeated injections at short intervals may be stressful to the animals<sup>2-4</sup>. Alternatives are providing the drug through drinking water which requires a ten-fold higher amount of buprenorphine due to the consumption and to first-pass hepatic extraction of orally applied buprenorphine. Furthermore, there is also the possibility of spillage of drinking water with most current caging systems. In addition, it remains unclear, whether individual animals voluntarily consume sufficient amounts of the medicated water to ensure permanent pain alleviation, especially during the daylight phase, when mice do not drink regularly. Nevertheless, providing analgesia with drinking water has become popular, particularly for short half-life opioids (e.g. Tramadol) or NSAIDs (e.g. acetaminophen/paracetamol).<sup>5</sup> Thus, long-lasting forms of analgesia are badly needed, however, sustained-release formulations of pain-killers are not commercially available to date in Europe, although those drugs have been developed for rodents, particularly buprenorphine which provides 1-3 days continuous pain treatment<sup>3, 6, 7</sup>.

Non-steroidal anti-inflammatory drugs (NSAID, e.g., meloxicam, carprofen) are often administered in mice; some of them are injected only 1-2 times per day based on the assumption that they provide pain relief for up to one day.<sup>1</sup> However current publications raise doubts about the analgesic efficacy of some NSAIDs. Particularly the efficacy of commonly used dosages and the suggested length of action remain unclear.<sup>8-10</sup> One of the problems arising from provision of pain relieving drugs to experimental animals is that these can interfere with the experiment. For example, in studies evaluating nociception or the effect of pain on a disease model, treatment with an analgesic may forfeit the purpose of the experiment. Similarly, when studying inflammatory diseases, medication including analgesics, may affect the inflammatory process itself and hence partially interfere with the

experimental goal.<sup>8</sup> These examples indicate that an evaluation of efficient pain management is difficult and may require certain compromises.

New indicators of pain are necessary to improve the quality of pain assessment. This is not simple to achieve and probably only the identification of behavioral traits can provide sufficient information during simple cage-side monitoring as performed when score sheets are used. For example, audible vocalizations cannot be unambiguously interpreted: are they due to real pain or distress? Recent studies, aimed at the identification of objective parameters for the assessment of pain, analyzed the link between behavioral changes and alterations in heart rate and heart rate variability using sensors implanted to continuously monitor physiological parameters.<sup>11, 12</sup> These experiments show good correlation of cardiovascular reactions with signs of pain and stress. Monitoring long-term physiological parameters and associated behaviors by continuous telemetric or video recordings (lasting for hours or days) provide evidence for pain only retrospectively. From a practical point of view such sensors cannot be implanted into each and every experimental animal, first because this would involve additional unnecessary stress and would undoubtedly be painful for some time after surgical implantation of the transmitter, but also because not all experimental situation would allow such a sophisticated operation e.g. in very young animals. Additionally, observation of alterations of specific spontaneous home-cage behavior such as burrowing and nest-building recently have been established not only as signs of disturbed wellbeing<sup>13</sup> or to monitor the progression of neurodegenerative diseases<sup>14-16</sup> but also for the detection of post-operative pain but may be time consuming<sup>16, 17</sup> Thus short cage-side observations taken immediately after the painful insult have been used for many years and allow the immediate treatment of pain. In practical terms, individuals are monitored for approximately 10 minutes either in the home cage or an observation area for very short-term aberrations of bodily appearance, that are known to be indicative for specific types of pain, e.g., press, stagger, stretch, twitch are thought to be typical signs of abdominal pain in mice<sup>18</sup> Such very specific symptoms are often combined to composite scales, summarizing the most frequent and indicative alterations<sup>19</sup> However, a well-known drawback of such cage-side observations is, that occurrence of aberrations provides some evidence for pain but absence of these during the short observation period does not prove that animals are pain-free.

Another approach to assess pain is the grimace scale: facial changes and contortions indicate pain<sup>20</sup>. This approach, although plausible, requires videotaping of individual animals and its use during routine monitoring is not yet validated.

The assessment requires adaptation to the experimental condition, animal strain, sex etc. and the observer needs training and experience to recognize and categorize facial features<sup>21</sup>.

Thus, methods reliably indicating pain are quite complicated needing intensive analysis of individual animals and additional technical devices. Behavioral science has implemented quantitative approaches, based on statistical analysis of animal cohorts. Assessment of individual animals, however, is more subjective and may be biased due to the investigators wish to continue without interfering with the experiment. Hence, implementation of robust surveillance protocols, which are quantitative, reproducible and allow longitudinal follow-up, are needed. Many investigators have

established their own 'score' sheet based on the requirements of the local animal ethics committee. These score sheets are usually not included or detailed in publications. Here we propose a score sheet for post-operative surveillance that includes a quantitative approach with cut-offs defining termination of the experiment and euthanasia of the animal in case of unacceptable suffering. We are well aware that this sheet is not exclusively based on scientific evidence but also on experience with several decades of animal experiments. The score sheet has been developed along with animal welfare officers and authorities in charge of ordinances for animal experimentation.

#### Animal models in abdominal surgery

Surgical modification of organs in the abdominal cavity include operations on the liver e.g. partial liver resection (see in <sup>22</sup>), liver transplantation<sup>23</sup>, bile duct ligation<sup>24</sup>. Partial or full splenectomies, kidney transplantation<sup>25</sup>, induction of strictures in the intestine, pancreatectomies<sup>26</sup>, pancreatic duct ligation<sup>27</sup> etc. are other examples of intra-abdominal interventions. These models have one thing in common: access to the abdominal cavity is by a laparotomy (incision through the abdomen). The post-operative time frame of these experiments can be between a few hours and several months, whereby pain is experienced in the first two-three days.

The most used procedure (e.g., partial hepatectomy) consists usually of a preoperative injection i.p. of buprenorphine (0.1 mg/kg body weight), approximately 30 minutes before the operation.

Subsequently, animals are shaved and immobilized under inhalation anesthesia using isoflurane. The animals are fixed by tape on a stainless steel plate in a supine position. The plate is warmed by a heating pad, maintaining 37-40°C. Eye ointment is applied and the abdomen is opened by a sagittal incision after disinfection with alcohol. To access the liver, the sternum may be pulled upwards and fixed by a 6-0 suture. The liver ligaments are then freed followed by individual ligation of vessels to liver lobes, which can then be dissected. After completion, the abdomen is then closed by a running suture of the peritoneal muscle/fascia followed by closing the skin. For recuperation, the animal is placed in a warming cabinet (28-30°C) for a few hours or overnight, prior to its return to the regular cage.

As in human surgery, the impact of the specific type of intervention (e.g. partial hepatectomy) may be moderate to severe and thus the access to the abdomen i.e. the laparotomy itself may have a variable impact on the overall procedure. Furthermore, a lot of procedures in human patients can be performed by laparoscopy (minimal invasive), which strongly reduces postoperative morbidity. Although minimal invasive approaches have been tested in rodents, routine use is not feasible.

In the current report, we focus predominantly on experiments concerning liver surgery. A classic example is a 70% resection of liver lobes<sup>22</sup>. This procedure is fairly standardized and benefits from the anatomy of the mouse or rat liver which are quite lobulated. Therefore, parenchymal transections are not required because the lobes can be individually removed by ligation of the vessels. This procedure is well tolerated. The liver usually regains its original size within a week. In addition, extended hepatectomies e.g. 86% and 91% removal of liver tissue, have been established to explore the effects of an insufficient liver volume<sup>28</sup>. This situation can lead to a so-called small-for-size syndrome: the

small liver is not able to regenerate, most probably due to the metabolic overload and concurrent accumulation of toxic products<sup>29</sup>.

The small-for-size syndrome, particularly with a full blown liver failure, results in reduced activity, apathy and encephalopathy ('hepatic coma'). Pain assessment of these animals has been debated, as reports from patients with similar syndrome do not complain of pain. In addition, analgesia may further impact on hepatic function and is given with caution.

#### Assessment of welfare

Usually, pain is assessed by the investigator who observes the animals for a short period of time looking predominantly at active behavior, ruffling of the fur and hunchback position. When there are several animals in a cage an individual repeated assessment over time is inaccurate. Once an animal is identified that is clearly in pain, it should be treated properly by analgesics. This leads to the question, should all animals in the experiment receive the same treatment to ensure consistency? Would it be better to treat all animals from the start with analgesic to avoid having to change the experimental protocol based on an individual animal? Alternatively, if a single animal shows abnormal behavior and pain, it might be better to eliminate the animal from the experiment. These are questions that should be clarified before beginning an experiment, particularly if the animals are valuable due to complex genetics or require special treatment e.g. diabetic mice receiving insulin.

Below we describe a score sheet used for monitoring after abdominal operations, particularly on the liver.

#### Post-operative score sheet

For each animal undergoing abdominal surgery, a score sheet is prepared, indicating animal ID, protocol ID, date and prospective severity degree (CH 0-III). Also the type of intervention should be indicated. This basic information will allow a replacement investigator take over surveillance, particularly if an animal caretaker or veterinarian has reported an animal ill.

Depending on the protocol, the severity or expected health issue, the investigator may be asked to supervise the animals very closely i.e. twice a day. For proper observation, it is advised to take each animal out of its cage, place it on top of the grid or lid as deemed best for observation of the animal. If there are only one or two animals per cage, observation may be performed without touching the animals, however, animals must be freely visible. Overall, a solid knowledge of normal behavior and appearance of a mouse of the respective line, sex and age is prerequisite for proper monitoring.

#### *Activity*

In the following, scored behaviors and signs of distress are described. Activity may be judged taking into account the time of day, which affects the diurnal activity pattern. Absence of activity is certainly an indicator of an impaired condition and health. However, normal or even increased activity is not necessarily an indicator for the absence of pain or suffering and it is mandatory to observe other parameters or symptoms to assess the picture. Aggressive animals may appear very active and aggression is a potential indicator of pain. Reduced activity is given one point while immobility is a strong factor and should be given three points. Increased activity is very subjective and might be induced by buprenorphine treatment<sup>3</sup> and therefore does not get any points in our score sheet. ..

### *Breathing*

Breathing rate and depth are not easy to assess in mice without specialized equipment. However, animals with flat breathing but normal frequency are given one point, while shallow, rapid breathing is an indicator for more severe physical impairment (two points).

### *Fur*

The appearance of fur (piloerection, ruffling) is perhaps the easiest parameter to judge. Ruffled, unclean or matted fur indicates absence of normal grooming behavior. This is either based on physical impairment (through the experiment) or a stress situation in the cage, particularly, if several males share a cage. In this case, presence of bites, wounds or aggressive behavior may indicate social imbalance. Such an imbalance can be prompted if one or two animals of the cohort are operated on and maybe weakened, resulting in a change of the social hierarchy. It is therefore recommended that operated animals should not be mixed with untreated, healthy litter mates for the time of recovery, i.e. during the first one to maximally four post-operative days only operated animals are kept together. Ruffled fur receives one point.

### *Posture*

Another, quite easy parameter is the posture, such as hunchback or arching behavior. This is observed when the animals are in strong abdominal pain and is found in combination with ruffled fur. Contradictory reports are present on the interpretation of hunched posture in mice<sup>18, 30-33</sup>, and it was recently described not to be a reliable indicator of pain<sup>18, 33</sup>. Based on our previous results on laparotomy<sup>11</sup> we assign one point for hunchback or arching behavior.

### *Jaundice*

Jaundice indicates loss of liver function and may be due to a direct interference with the liver by surgery or pharmacological treatments targeting the liver. It appears as a yellowing of the skin. In case of mice with black or dark fur, the eyes or the footpads easily allow an assessment. If jaundice is part of the pathophysiology of the model, bilirubin should be measured regularly by blood analysis. If another organ is targeted, blood parameters can be assessed: creatinine for kidney, amylase for pancreas, T3/4 for thyroid etc. This parameter receives one point.

### *Operative site: Assessment of the wound*

Wound infections are rare in rodents. In contrast to humans and larger animal species, particularly pets, the wound cannot be covered by a wound dressing. It is therefore crucial that the wound be properly closed to avoid entry of foreign material, or worse, extrusion of an intestinal loop. For proper postoperative examination, particularly in the first two days, mice have to be removed from the cage and individually inspected.

Particularly when pain medication is insufficient mice tend to bite and gnaw at their wounds, further enhancing chances for infection. However, overdosing with buprenorphine might also lead to aberrant behavior where the animals mutilate the wound (e.g. removal of sutures).

An open or infected wound receives two points. Additional criteria specific to the experiment can be introduced and scored.

### *Overall score*

At conclusion of an assessment, points should be added up. A mouse having two or more points should receive pain treatment. In the case of five points, a critical physical impairment has been

reached which should receive particular attention. Adequate pain management should be provided in both cases, i.e., a protocol (giving detailed advice on drug, dosage, administration route and interval) must be in place, which has been developed and tailored regarding the model (e.g., interference with experimental goals) and type of pain. Typically, we administer a bolus injection of buprenorphine (0.1 mg/kg s.c.) two or three times a day. If no improvement is observed within the next 24 hours, the animal should be euthanized. Below is a summary of the criteria leading to termination. Some are independent criteria e.g. cachexia (short and long term weight loss) or if the animal is self-mutilated.

Summary of termination criteria:

- the animal does not recuperate from the intervention (immobile, unresponsive to stimuli 2 hrs after the operation)
- **score  $\geq 6$  points.**
- **Score = 5 points:** plus no improvement within the next 24 hrs
- the animal loses  $>15\%$  of body weight within 12 hours after operation (short term weight loss)
- the animal loses  $>20\%$  of body weight compared to start of experiment (long term weight loss)
- animal is self-mutilated
- animal exhibits signs of pain despite buprenorphin treatment for three days.
- animal does not respond to pain treatment within 24 hrs

#### Refinement opportunities

Through an invasive procedure of the abdominal cavity, an animal is transiently incapacitated and may lose its social status. It is imperative that the animal has opportunity to create a refuge in the form of nest building or burrowing material or shelters. Particularly in male animals, short-term isolation during intervention, and physical impairment may severely disturb the social structure. It should be evaluated whether a single individual should be placed back in the cage with its healthy littermates. Presumably, if all animals in a cage are operated at the same time, the individual will suffer less from the possibly disrupted social structure or may even profit from social support<sup>3</sup>.

#### Recommendation and Further 3Rs research

Historically, surgeons preferred to operate on male mice. With their tendency to aggressive behavior, increasing with age, female mice might be more amenable. In particular, studies addressing age-related topics would greatly benefit from the inclusion of female mice. The social structure of the rat is different and males are less aggressive. Here, the need to focus on one sex is not as crucial. In both species, it might be advisable in the future to use both genders. First, to exclude conclusions that may or may not apply to both sexes, and second, the use of all animals reduces the overall number of animals to be produced. Currently, a vast number of animals are still killed since the demand for one sex is much bigger for certain animal models.

#### **Conclusion**

Proper animal surveillance in routine settings is only possible if the process is easy and quick. Otherwise the likelihood that investigators will not seriously assess the wellbeing of their animals' increases. This is to the detriment of the animal. It may ultimately affect the experimental outcome



negatively and prevent relevant conclusions. The lack of diligence may also be a result of mice or rats observed to 'happily' and actively run around the cage. Such activity may imply that these animals are not in pain. This misconception is still commonly observed in many investigators and should be rectified by education and training.

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